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The *cop* operon is required for copper homeostasis and contributes to virulence in *Streptococcus pneumoniae*

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Summary

High levels of copper are toxic and therefore bacteria must limit free intracellular levels to prevent cellular damage. In this study, we show that a number of pneumococcal genes are differentially regulated by copper, including an operon encoding a CopY regulator, a protein of unknown function (CupA) and a P1-type ATPase, CopA, which is conserved in all sequenced *Streptococcus pneumoniae* strains. Transcriptional analysis demonstrated that the *cop* operon is induced by copper *in vitro*, repressed by the addition of zinc and is autoregulated by the copper-responsive CopY repressor protein. We also demonstrate that the CopA ATPase is a major pneumococcal copper resistance mechanism and provide the first evidence that the CupA protein plays a role in copper resistance. Our results also show that copper homeostasis is important for pneumococcal virulence as the expression of the *cop* operon is induced in the lungs and nasopharynx of intranasally infected mice, and a *copA*$^-$ mutant strain, which had decreased growth in high levels of copper *in vitro*, showed reduced virulence in a mouse model of pneumococcal pneumonia. Furthermore, using the *copA*$^-$ mutant we observed for the first time in any bacteria that copper homeostasis also appears to be required for survival in the nasopharynx.

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Introduction

Streptococcus pneumoniae is the main cause of bacterial pneumonia worldwide and is also a major agent of otitis media, bacteraemia and meningitis. For *S. pneumoniae* to survive and cause infection in the very different host environments associated with these diseases, it must be able to sense and adapt to considerable variation in environmental conditions, including changes in the concentration of metal ions in host tissues.

Transition metal ions are essential co-factors for many enzymes but they can also be highly toxic. Therefore, bacterial metal ion homeostasis is extremely important to ensure sufficient intracellular levels of metal ions for use as co-factors, but also to limit excess intracellular levels to prevent toxicity. Several metal ion transport systems have been implicated in pneumococcal virulence (Brown *et al.*, 2001; McAllister *et al.*, 2004; Hendriksen *et al.*, 2009; Rosch *et al.*, 2009), and furthermore the concentration of metal ions can influence virulence gene expression (Johnston *et al.*, 2006; Kloosterman *et al.*, 2007; Gupta *et al.*, 2009; Shafeeq *et al.*, 2011). Under conditions of limitation, metal ions are imported into *S. pneumoniae* by specific metal ion transporters such as PsaBCA, PitABCD and AdcCBA, which are responsible for the transport of manganese, iron and zinc respectively (Dintilhac *et al.*, 1997; Dintilhac and Claverys, 1997; Brown *et al.*, 2001; Johnston *et al.*, 2004; Shafeeq *et al.*, 2011), while an excess of metal ions is removed from the cell by specific efflux systems, for example the zinc exporter CzcD (Kloosterman *et al.*, 2007) and the manganese exporter MntE (Rosch *et al.*, 2009).

Copper is also an important transition metal for most organisms, albeit toxic at high levels. So far, copper homeostasis has not been studied in *S. pneumoniae*. Like in many Gram-positive bacteria, known copper-containing proteins have not been identified in the *S. pneumoniae* genome sequences (Solioz *et al.*, 2010). However, there is some evidence that pneumococci may require copper, as a *S. pneumoniae* strain mutant for the (p)ppGpp synthetase *relA* is unable to grow in chemically defined medium (CDM) unless it is supplemented with copper and

manganese (Kazmierczak *et al*., 2009). Even if copper is not used, pneumococci must still have mechanisms to export an excess of copper from the cell, as *S. pneumoniae* will encounter varying levels of copper *in vivo*. In some tissues, especially in the blood, free copper levels are very low, but levels can be higher in tissues, for example the lungs (lungs 121.96 and blood 12.98 $\mu g\ g^{-1}$ dry weight, Catalani *et al*., 2008). Moreover, copper levels in the serum have been shown to increase during infection (Arredondo and Nunez, 2005).

To maintain copper homeostasis and prevent toxicity, bacteria use a number of efflux and sequestration mechanisms to remove excess copper, and also initiate a global adaptive genetic response which can involve induction of other stress regulons (Kershaw *et al*., 2005; Teitzel *et al*., 2006; Ward *et al*., 2008; Baker *et al*., 2010). However, the cause of toxicity, the mechanisms of resistance and the regulatory responses used can vary significantly between species. Resistance mechanisms include efflux systems, such as the ubiquitous CopA/CopB P1-type ATPase transporters (Veldhuis *et al*., 2009), and sequestration mechanisms, including the CopZ family of copper binding proteins, which chaperone the copper ions intracellularly for incorporation/use/efflux by other copper-binding proteins (Portmann *et al*., 2006). Two main types of Gram-positive copper-responsive regulators have been identified to date. These are the CopY copper-responsive repressor family found in *Enterococcus* and *Streptococcus* spp. (Portmann *et al*., 2006), and the CsoR copper-responsive repressors found in *Mycobacterium tuberculosis* (Liu *et al*., 2007) and *Bacillus subtilis* (Smaldone and Helmann, 2007).

The objective of this study was to investigate the mechanism of pneumococcal copper homeostasis and its role in virulence. Transcriptional profiling with DNA microarrays identified a number of genes that are differentially expressed depending on the copper concentration, including a conserved tri-partite operon encoding a homologue of the CopY regulator family (*copY*), a hypothetical protein (*cupA*) and a P1-type ATPase (*copA*). Transcriptional analysis by real-time quantitative reverse transcription (RT)-PCR and assaying strains carrying transcriptional *lacZ* fusions demonstrated that expression of the *cop* operon is induced specifically by copper *in vitro* and is autoregulated by CopY. Expression of the *cop* operon, as well as several other putative copper transport genes, is also induced in pneumococci isolated from the lungs and nasopharynx of intranasally infected mice. Furthermore, a *copA*$^-$ mutant strain showed decreased virulence in a mouse model of pneumococcal pneumonia and a decreased ability to survive in the mouse nasopharynx, showing that copper homeostasis plays an important role in *S. pneumoniae* physiology and virulence.

Results

S. pneumoniae *encodes a number of putative copper homeostasis genes*

Bioinformatic analysis showed that all sequenced pneumococcal genomes contain an operon with homology to the *cop*-like operon of *Enterococcus* and other species of the *Lactobacillale* order (Reyes *et al*., 2006). The *S. pneumoniae cop*-like operon encodes a putative copper-dependent repressor protein (designated *copY*) and ATPase (designated *copA*), which are conserved in all other *Lactobacillale*, as well as a gene of unknown function which is unique to *S. pneumoniae*, *Streptococcus mitis* and *Lactobacillus johnsonii* (Reyes *et al*., 2006) (Fig. 1A). Analysis of the flanking regions of the *cop*-like operon identified −10 and −35 promoter sequences in the upstream region of *copY* and a possible terminator sequence downstream of *copA* (Fig. 1A). RT-PCR using intergenic primer sets confirmed that the three genes form an operon and are transcribed as a single transcript, as is the case in *Enterococcus* (Fig. 1B, Solioz and Stoyanov, 2003).

The *S. pneumoniae* CopY (SPD0633) polypeptide (Fig. 1A) has 33% identity and 64% similarity to *Enterococcus hirae* CopY at the amino acid sequence level. The N-terminal domain has all the conserved amino acids of the 'winged' helix DNA binding motif found in other CopY proteins, but appears to have a truncated C-terminus and only has the first CxC part of the $CxCx_{4-6}CxC$ copper-binding domain found in other CopY homologues (Portmann *et al*., 2006). The annotated translational start sites for *copY* differ between genome sequences. However, analysis of the nucleotide sequences shows that there is 100% homology in this region, suggesting that the genomes with *copY* translational start sites different to the D39 sequence shown in Fig. 1A are annotated incorrectly. *In silico* analysis identified two *lactobacillale* CopY binding motifs (TACAnnTGTA) in the *S. pneumoniae* D39 *copY* promoter region (Fig. 1A, Reyes *et al*., 2006), suggesting that the *S. pneumoniae cop* operon is autoregulated by CopY.

The *copA* gene (*SPD0635*) encodes a P1-type ATPase, which has 35–44% amino acid sequence identity to other Gram-positive ATPases involved in the efflux of copper (Reyes *et al*., 2006). The predicted CopA polypeptide contains three conserved domains; an amino terminal plastocyanin-like domain, an E1-E2 ATPase domain and a carboxyl terminal haloacid dehalogenase-like hydrolase domain. Unlike *copY* and *copA*, the third gene (*SPD0634*) in the *S. pneumoniae cop*-like operon is not highly conserved between species (Reyes *et al*., 2006). *SPD0634* is predicted to encode a 123-amino-acid protein of unknown function, which has a plastocyanin or cupredoxin-like domain which may bind copper, and hence, has been designated *cupA*.

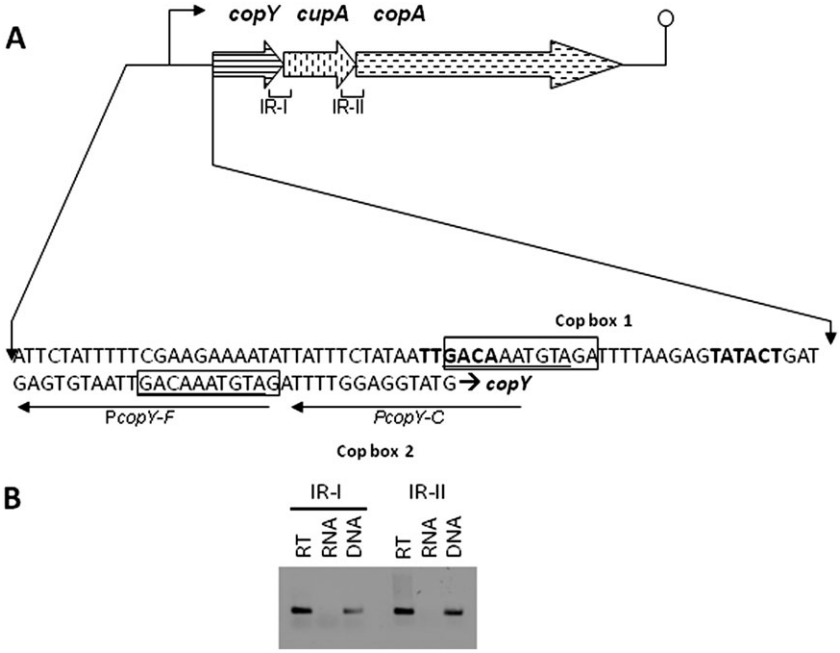

Fig. 1. A. The structure of the *S. pneumoniae cop* operon encoding *copY*, a putative copper transcriptional regulator, *cupA*, a hypothetical protein and *copA*, a copper-translocating P-type ATPase. The location of the putative promoter and terminator are indicated by an arrow and circle respectively. Nucleotides in bold indicate the putative core promoter sequences and boxed nucleotides indicate putative *cop* box consensus sequences. Arrows indicate the sequence of PcopY-C and PcopY-F reverse primers used to construct P*copY-wt–lacZ* and P*copY-mut–lacZ*.
B. Reverse transcriptase PCR analysis to confirm the polycistronic nature of the *S. pneumoniae cop* operon. RT-PCR was performed on total RNA isolated from D39 wild-type grown in CDM + 0.05 mM Cu^{2+} with (RT) and without (RNA) reverse transcriptase treatment using the IR-I and IR-II intergenic region primer pairs (see Fig. 1A). DNA was used as a positive control.

Interestingly, unlike many other Gram-positives (Gaballa and Helmann, 2003; Solioz and Stoyanov, 2003; Sitthisak *et al*., 2007), *S. pneumoniae* does not encode a *copZ* copper chaperone. In Gram-positives, copper is usually donated to CopY by CopZ (Cobine *et al*., 1999; 2002). Therefore, it is not clear how copper is chaperoned or how the putative copper-dependent regulator CopY senses copper in pneumococci. *In silico* analysis only identified three other genes with homology to copper homeostasis proteins in the sequenced *S. pneumoniae* genomes – two P-type ATPase genes: *ctpE* (*SPD1927*) and *ctpC* (*SPD1436*); and gene *cutC* (*SPD1118*), which exhibits 34% amino acid identity and 53% similarity to the *E. coli cutC* gene. CutC is required for maximal copper tolerance in *E. coli* (Gupta *et al*., 1995) and is conserved in the *Lactobacillale*, suggesting that it has an important function in these bacteria (Reyes *et al*., 2006).

The copY *promoter is induced by copper, repressed by zinc and auto-repressed by CopY*

The transcriptional response of the *cop* operon to selected metal ions was investigated using *S. pneumoniae* D39 strain carrying an ectopic P*copY–lacZ* fusion integrated into the *bgaA* gene, which contained the entire *copY* promoter region. Specific β-galactosidase activity from the putative promoter was examined in CDM containing different concentrations of various metal ions: Zn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} and Cu^{2+}. As seen in Fig. 2A, only Cu^{2+} ions caused induction of P*copY–lacZ* expression in *S. pneumoniae*. Further analysis of Cu^{2+} responsiveness of the promoter showed that there was a gradual response with increasing concentrations of added Cu^{2+} ions (Fig. 2B), with the highest level of expression observed at 0.05 mM Cu^{2+}, which is a concentration of Cu^{2+} that does not inhibit growth (Fig. 4).

The metal ion specificity of the *copY* promoter was further investigated by growing pneumococci with a constant amount of copper and various concentrations of other metal ions: Zn^{2+}, Co^{2+}, Ni^{2+} and Fe^{2+} (Fig. 3A). Addition of up to 0.05 mM Co^{2+} or Ni^{2+} had no effect on promoter activity. However, 0.1 and 0.5 mM Fe^{2+} caused a 1.4-fold increase ($P < 0.01$) and addition of 0.1 mM Zn^{2+} resulted in a twofold decrease ($P < 0.01$) in promoter activity compared with that in the absence of any metal ions. The addition of increasing concentrations of zinc to various constant amounts of copper resulted in a concentration-dependent decrease in the expression of P*copY–lacZ* (Fig. 3B), suggesting that, as in *E. hirae* (Cobine *et al*., 1999; 2002), zinc may act as a CopY co-repressor in *S. pneumoniae*.

To elucidate whether the *cop* operon is regulated by CopY, a *copY-stop* mutant derivative of D39 was constructed. Since *copY* is the first gene of the operon, a non-polar knock out was obtained through insertion of four premature stop codons in the *copY* coding sequences, in order to disrupt CopY translation, but not transcription of the *cop* operon. Transcriptional analysis showed that expression of P*copY–lacZ* was completely de-repressed in the *copY* mutant compared with the wild-type strain, demonstrating that the *cop* operon is auto-repressed by CopY (Fig. 3B and C). Increased expression of P*copY–lacZ* in the *copY* mutant compared with the wild-type strain in the presence of Cu^{2+}, demonstrates that

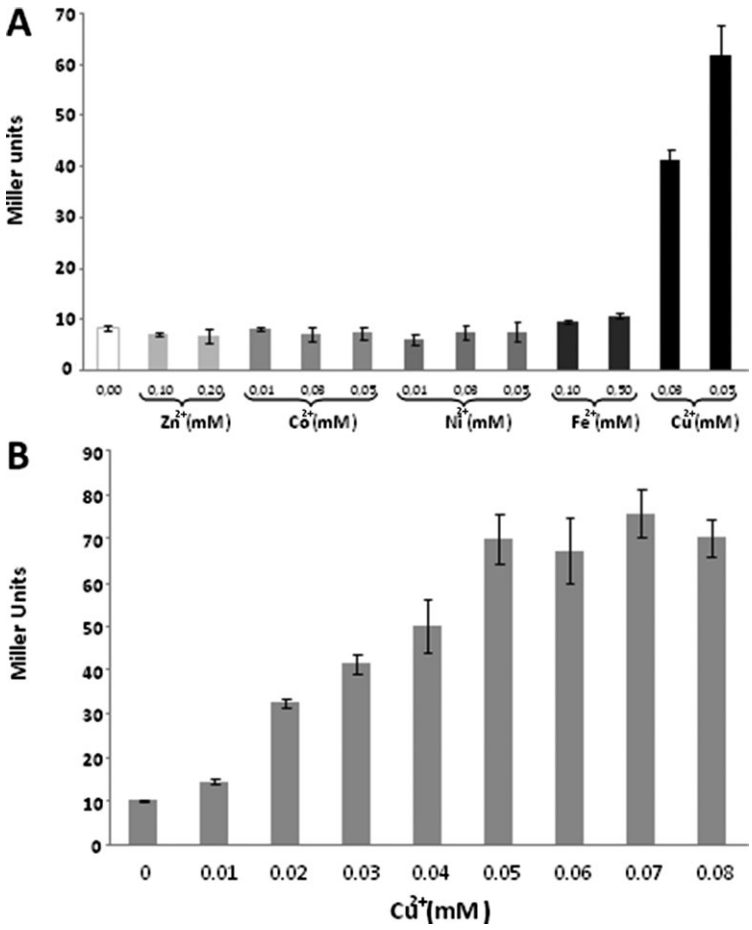

Fig. 2. Expression levels (in Miller units) of a P*copY–lacZ* transcriptional fusion in D39 wild-type in CDM (without copper addition): (A) supplemented with various divalent metal ions; (B) supplemented with different concentrations of Cu^{2+}. Standard deviations of three replicates (A) or independent experiments (B) are indicated on each bar.

there is a substantial level of CopY repression of the *cop* operon in the wild-type, even in the presence of Cu^{2+}. In the *copY*-stop mutant, the addition of zinc did not repress P*copY–lacZ* activity as seen in wild-type pneumococci (Fig. 3C). Complementation of the *copY* gene in the *copY*-stop mutant strain led to restoration of the repression and Cu^{2+}-responsiveness of P*copY* in the *copY*-stop mutant (Fig. 3C). Overall, these results show that the *S. pneumoniae cop* operon is specifically induced by copper and is autoregulated by the CopY repressor protein.

Two sequences similar to the *Lactobacillale* cop box consensus sequence are found in the *copY* promoter region of *S. pneumoniae* D39 (Fig. 1A). Cop box 1 is distal to the translational start sequence and overlaps with the predicted core promoter, while cop box 2 is present just upstream of the putative ribosomal binding site (RBS). The role of the CopY binding motif in copper-responsive gene expression was investigated using *S. pneumoniae* D39 strains carrying P*copY–lacZ* fusions that contained both cop boxes (P*copY-wt*) or with cop box 2 deleted (P*copY-mut*), which was expected not to interfere with the integrity of the core promoter. Specific β-galactosidase activity from the two promoters was compared in CDM supplemented with 0.05 mM Cu^{2+}. As seen in Fig. 3D, the absence of cop box 2 in P*copY-mut* resulted in a significant increase in β-galactosidase activity to the level observed with the wild-type promoter (P*copY-wt*) in the *copY-stop* mutant strain. In the *copY-stop* mutant, there was no significant difference observed between the wild-type and mutant promoters. Thus, these results show that CopY repression occurs through the cop box sequence identified in the *cop* operon promoter.

CopA is required for pneumococcal copper resistance in vitro

To investigate the physiological function of the *cop* operon genes in *S. pneumoniae*, isogenic *copY-stop, cupA-stop*, *ΔcopA* and *ΔcupA copA* mutants were constructed and characterized *in vitro*. Growth assays of wild-type D39 and the mutants in GM17 supplemented with various metal ions demonstrated that none of the strains tested appear to require Cu^{2+} for growth in these conditions (Fig. 4A). However, copper is toxic to pneumococci in high concentration as 1 mM Cu^{2+} was inhibitory to growth

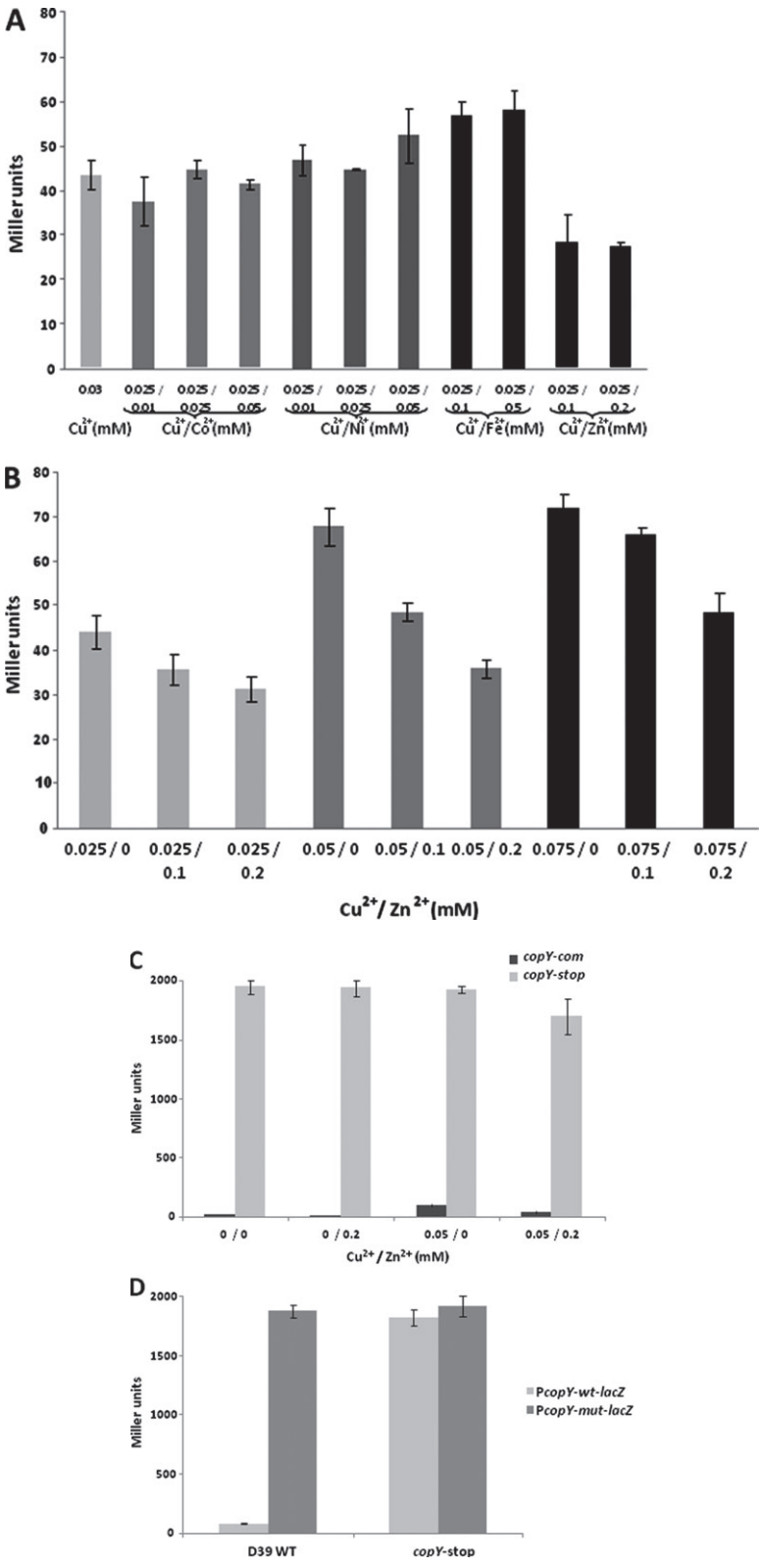

Fig. 3. Expression levels (in Miller units) of a P*copY–lacZ* transcriptional fusion in D39 wild-type in CDM (without copper and zinc addition): (A) supplemented with various combinations of copper and zinc concentrations; (B) supplemented with 0.025 mM Cu^{2+} and with different concentrations of other divalent metal ions. Standard deviation of three independent experiments or replicates is indicated on each bar.
C. Expression levels (in Miller units) of a P*copY–lacZ* transcriptional fusion in D39 *copY*-stop and D39 *copY*-stop-com in CDM (without copper and zinc addition) supplemented with various combinations of copper and zinc concentrations. Standard deviation of three independent experiments is indicated on each bar.
D. Expression levels (in Miller units) of a P*copY-wt–lacZ* and P*copY-mut–lacZ* transcriptional fusions in D39 wild-type and D39 *copY*-stop in CDM supplemented with 0.05 mM Cu^{2+}. Standard deviation of three independent experiments is indicated on each bar.

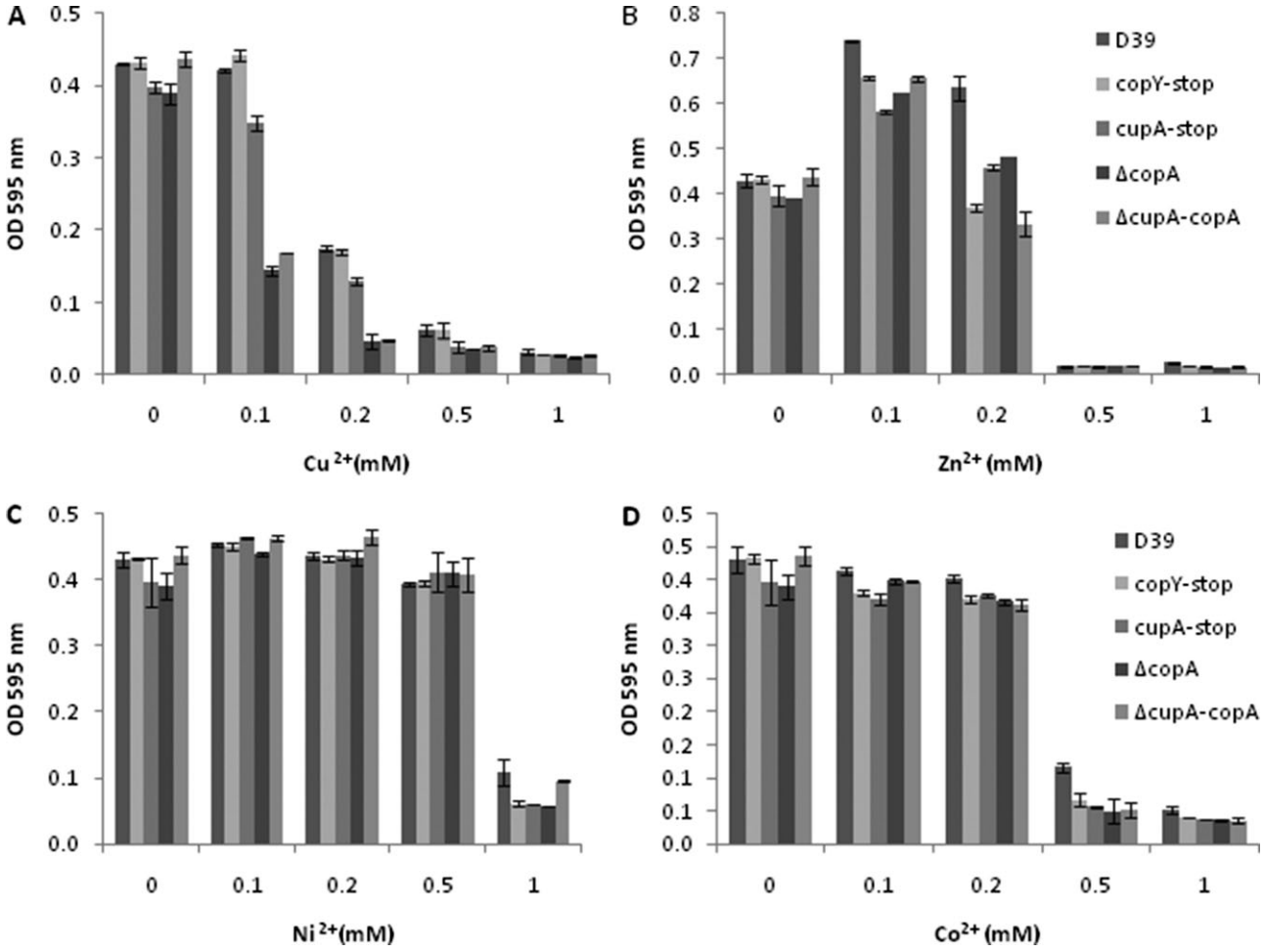

Fig. 4. Growth of *S. pneumoniae* strains D39 and the isogenic *copY-stop*, *cupA*-stop, Δ*copA* and *cupA-copA*$^-$ mutants after 8 h of growth in GM17 with increasing concentrations of Cu^{2+} (A), Zn^{2+} (B), Ni^{2+} (C) or Co^{2+} (D). Optical densities at 595 nm were determined after 8 h of growth. Results represent the mean and standard deviation of three independent experiments.

(Fig. 4A). In contrast, both the single *ΔcopA* and the double *ΔcupA copA* mutants showed significant inhibition of growth at 0.1 mM Cu^{2+} ($P < 0.0$, Fig. 4A) and therefore demonstrated significantly decreased resistance against Cu^{2+} compared with the wild-type D39 and the *copY-stop* mutant. The *cupA-stop* mutant was also more sensitive to Cu^{2+} than the wild-type ($P < 0.01$), although not to the same level as the *ΔcopA* single and double mutant suggesting that while the CupA copper binding protein plays a role in pneumococcal copper tolerance, the CopA ATPase is the major copper resistance mechanism.

The comparative growth of the wild-type D39 and *copY-stop* mutant and the difference in growth between the *cupA-stop* and *copA* mutants show that the translational stop mutations in the *copY* and *cupA* genes do not prevent the expression of the downstream genes. The comparative growth of the *copY-stop* mutant and wild-type D39 is expected as de-repression of the copper efflux systems in the *copY-stop* mutant would be comparable to the induced response in the wild-type. No significant differences in growth were observed for any of the strains when grown with various concentrations of Co^{2+} and Ni^{2+} (Fig. 4C and D). However, wild-type D39 appears to grow significantly better than all the *cop* operon isogenic mutants in 0.1 and 0.2 mM Zn^{2+} ($P < 0.01$), indicating that the *cop* operon may play a role in resistance to excess levels of Zn^{2+} as well as Cu^{2+}. There was no significant difference in the doubling times of the strains in the absence of added metal ions or in the presence of low copper/other metal ions (Zn, Ni and Co) as compared with the wild-type except for the *cupA, copA* and *cupA-copA* mutants, which were increased in the presence of an increasing copper concentration (Fig. 4). Eight-hour endpoints were taken as the important differences between all strains were most clearly visible after 8 h of growth and no autolysis was observed at this time point. Therefore, together, our data show that CopA is specifically required for resistance to copper.

ICP-MS analysis with and without added 0.05 mM Cu^{2+}

To determine whether the transcriptional effects observed in high and low copper microarray correlate with a cell-associated concentration of Cu^{2+}, ICP-MS analysis was performed on cells grown in CDM medium without and with 0.05 mM added Cu^{2+}. ICP-MS analysis revealed that *S. pneumoniae* D39 grown in the presence of added Cu^{2+} has a 13-fold higher cell-associated amount of Cu^{2+} as compared with D39 grown in the absence of added Cu^{2+} (13 μg g^{-1} dry mass of cells versus > 1 μg g^{-1} dry mass of cells). These results indicate that the transcriptome effects observed above are mainly due to the difference in intercellular Cu^{2+} concentration.

To determine the role of CopA, the *copA* mutant was grown in CDM medium with 0.05 mM added Cu^{2+}. ICP-MS analysis revealed high accumulation of intracellular Cu^{2+} in the CopA mutant as compared with the D39 wild-type in the presence of 0.05 mM added Cu^{2+} (65 μg g^{-1} dry mass of cells versus 13 μg g^{-1} dry mass of cells. High accumulation of Cu^{2+} in the *copA* mutant suggests the function of CopA as a Cu^{2+} efflux transporter in *S. pneumoniae*.

Identification of copper regulated genes in S. pneumoniae

To investigate the effect of copper on global gene expression in *S. pneumoniae* and the role that CopY plays in that response, the transcriptomes of (i) D39 wild-type grown in CDM with and without copper, and (ii) D39 wild-type and D39 *copY* mutant strains in low copper were compared. For (i), a concentration of 0.05 mM Cu^{2+} was used as this concentration does not significantly affect growth but activates P*copY* to a high degree (Figs 2 and 5). The amount of zinc in CDM was decreased to 5 μm from the standard concentration of 17.5 μm, in order to prevent Zn^{2+} repression of copper responsive genes as was seen in Fig. 3.

Tables 1 and 2 summarize the transcriptomic changes in *S. pneumoniae* induced by copper excess compared with limitation, in D39 wild-type. After applying the criteria of ≥ twofold difference as the threshold change and a *P*-value of < 0.001, 39 genes were differentially expressed, of which 22 were upregulated and 17 were downregulated in the presence of copper. The *copY* (SPD0633), *cupA* (SPD0634) and *copA* (SPD0635) genes showed the highest level of expression in high copper conditions, confirming the β-galactosidase assays (Figs 2 and 3) and also demonstrating that the microarray results are a valid representation of the pneumococcal global response to excess copper (Table 1). Strikingly, the transcriptional profiling did not identify any of the known copper homeostasis homologues mentioned above (*ctpC, ctpE* and *cutC*). However, several genes displayed increased expression in the presence of copper, including: a number of putative

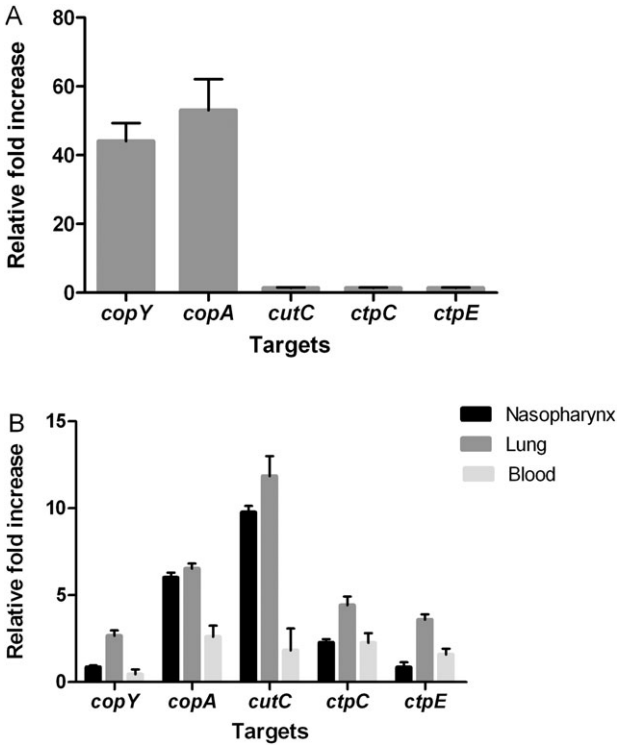

Fig. 5. The relative *in vitro* and *in vivo* expression of pneumococcal genes linked to copper metabolism.
A. *S. pneumoniae* strain D39 was grown in Sicard's defined medium *in vitro*, in the presence or absence of 0.05 mM Cu^{2+}. The expression of the genes *copY*, *copA*, *cutC*, *ctpC* and *ctpE* was normalized with housekeeping gene *gyrB*. Results represent the mean and standard deviation of three independent experiments. The fold increase is relative to the expression in Sicard's in the absence of copper.
B. Total RNA was extracted from D39 pneumococci isolated from the nasopharynx (black bars), lungs (dark grey bars) and blood (light grey bars) of intranasally infected MFI mice. The expression of the genes *copY*, *copA*, *cutC*, *ctpC* and *ctpE* was normalized with housekeeping gene *gyrB*. Results represent the mean and standard deviation of three independent experiments. The fold increase is relative to the expression in Sicard's in the absence of copper *in vitro*.

exoglycosidases such as the surface-associated β-*N*-acetyl glucosaminidase (SPD0444) and *strH*, which is an important pneumococcal virulence factor (King *et al.*, 2006); a putative operon encoding an uncharacterized transcriptional regulator (SPD1565) and a putative thioredoxin, and several amino acid transporters (Table 1). Unlike the effect of copper in other Gram-positive bacteria, such as *M. tuberculosis* (Ward *et al.*, 2008) and *Staphylococcus aureus* (Baker *et al.*, 2010), there was no induction of any genes known to be involved in oxidative stress resistance or the misfolded protein response, showing that *S. pneumoniae* responds differently to copper than other bacteria.

Genes downregulated in the presence of copper included several constituents of the purine metabolism pathway, SPD0051, SPD0058, SPD0059, SPD1628 and

Table 1. Summary of upregulated genes in transcriptome comparison of *S. pneumoniae* strain D39 grown in CDM plus 0.05 mM Cu^{2+} and CDM plus 0 mM Cu^{2+}.

TIGR4 locus tag	D39 locus tag	Function (TIGR4 annotation)	Ratio[a]
SP0057	SPD0063	*N*-acetyl-beta-hexosaminidase (carbohydrate metabolism)	2.7
SP0090	SPD0088	ABC transporter (polysaccharide), permease protein	4.4
SP0148	SPD0150	ABC transporter, substrate-binding protein	2.1
SP0409	SPD0373	Hypothetical protein (carboxymuconolactone decarboxylase family)	3.3
SP0498	SPD0444	Endo-beta-N-acetylglucosaminidase (carbohydrate metabolism)	3.1
SP0620	SPD0540	Amino acid ABC transporter, amino acid-binding protein	2.1
SP0709	SPD0616	(Polar) amino acid ABC transporter, ATP-binding protein	2.1
SP0710	SPD0617	(Polar) amino acid ABC transporter, permease protein	2.7
SP0711	SPD0618	(Polar) amino acid ABC transporter, permease protein	3.3
SP0727	SPD0633	Putative copper responsive regulator	19.0
SP0728	SPD0634	Hypothetical protein	19.1
SP0729	SPD0635	Putative copper-transporting P-type ATPase	11.6
SP1774	SPD1565	Transcriptional regulator (ArsR family)	1.5
SP1775	SPD1566	Hypothetical protein	2.3
SP1776	SPD1567	Thioredoxin	2.0
SP2072	SPD1899	Glutamine amidotransferase, class-I	2.7
SP2132	SPD1962	Hypothetical protein	3.7
SP2133	*	Hypothetical protein	2.8
SP2141	SPD1969	Glycosyl hydrolase-related protein (aminosugars metabolism)	3.3
SP2142	SPD1970	ROK family protein (transcriptional regulator/sugar kinase, NagC)	4.4
SP2143	SPD1971	Alpha-mannosidase (carbohydrate metabolism)	3.2
SP2144	SPD1972	Hypothetical protein	4.2

a. Ratios $\geq$ 2.0 (D39 wild-type + 0.05 mM Cu^{2+} compared with D39 wild-type + 0 mM Cu^{2+}). All *P*-values are < 0.001.
In case of putative operons neighbouring genes with ratios < 2.0 are also indicated.
* Not annotated in NCBI database, despite 100% DNA sequence identity to TIGR4 SP2133.

SPD1629, an operon encoding proteins involved in glycerophospholipid metabolism, as well as a number of ABC transporters (Table 2). Several hypothetical proteins, without predicted functions, were also among the differentially expressed genes.

The transcriptome analysis of the *copY*-stop mutant strain showed strong upregulation of the *cop* operon compared with the wild-type strain, thus confirming the autoregulation of this transcriptional unit (Table 3). Other genes affected by the *copY-stop* mutation include genes

Table 2. Summary of downregulated genes in transcriptome comparison of *S. pneumoniae* strain D39 grown in CDM plus 0.05 mM Cu^{2+} and CDM plus 0 mM Cu^{2+}.

TIGR4 locus tag	D39 locus tag	Function (TIGR4 annotation)	Ratio[a]
SP0044	SPD0051	Phosphoribosylaminoimidazole-succinocarboxamide synthase (purine metabolism)	–34.1
SP0051	SPD0058	Phosphoribosylamine-glycine ligase (purine metabolism)	–4.7
SP0053	SPD0059	Phosphoribosylaminoimidazole carboxylase catalytic subunit (purine metabolism)	–2.3
SP0112	SPD0109	Amino acid ABC transporter, periplasmic amino acid-binding protein	–2.2
SP0113	SPD0110	Argininosuccinate synthase	–2.7
SP0287	SPD0267	Xanthine/uracil permease family protein	–2.6
SP0585	SPD0510	Homocysteine methyltransferase (methionine metabolism)	–3.2
SP1027	SPD0913	Inosine-5′-monophosphate dehydrogenase	–2.1
SP1127	SPD1010	Hypothetical protein	–2.2
SP1695	SPD1506	Acetyl xylan esterase	–2.3
SP1696	*	Hypothetical protein	–5.4
SP1826	SPD1609	ABC transporter, substrate-binding protein	–2.0
SP1847	SPD1628	Xanthine phosphoribosyltransferase (purine metabolism)	–4.7
SP1848	SPD1629	Xanthine permease	–2.8
SP2184	SPD2011	Glycerol uptake facilitator protein	–2.1
SP2185	SPD2012	Hypothetical protein (glycerophospholipid metabolism)	–2.0
SP2186	SPD2013	Glycerol kinase, GlpK (glycerophosolipid metabolism)	–2.4
SP2240	SPD2069	Sporulation protein, SpoJ	–2.2

a. Ratios $\leq$ –2.0 (D39 wild-type + 0.05 mM Cu^{2+} compared with D39 wild-type + 0 mM Cu^{2+}). All *P*-values are < 0.001
In case of putative operons neighbouring genes with ratios > –2.0 are also indicated.
* Not annotated in NCBI database, despite 100% DNA sequence identity to TIGR4 genome.

Table 3. Genes differently expressed in D39 *copY*-stop strain grown in CDM with no added Cu^{2+}.

TIGR4/R6 locus tag	D39 locus tag	Function (TIGR4 annotation)	Ratio[a]
SP0303	SPD0277	6-phospho-beta-glucosidase (starch and sucrose metabolism)	2.6
SP0306	SPD0280	Putative transcriptional regulator, BglG	2.4
SP0308	SPD0281	PTS system, IIA component, CelC	1.5
SP0309	SPD0282	Hypothetical protein	2.7
SP0727	SPD0633	Putative copper responsive regulator	59.7
SP0728	SPD0634	Hypothetical protein	42.9
SP0729	SPD0635	Putative copper-transporting P-type ATPase	30.0
SPR1309	SPD1284	Hypothetical protein	–3.0
SP1434	SPD1263	ABC transporter, ATP-binding / permease protein	–2.0
SP1435	SPD1264	ABC transporter, ATP-binding / permease protein	–1.6
SP1436	SPD1265	Hypothetical protein	–1.3

a. Ratios ≥ 2 or ≤ –2.0 (D39 *copY*-stop compared with D39 wild-type). All *P*-values are < 0.001.
In case of putative operons neighbouring genes with ratios < 2.0 and > –2.0 are also indicated.

participating in cellobiose utilization (SPD0277, SPD0280 and SPD0282), which were upregulated in the *copY*-stop mutant. Four genes were downregulated (SPD1284, SPD1263-5), two of which encode a putative ABC transporter. Surprisingly, very few of the copper- responsive genes described in Tables 1 and 2 appeared to be affected by the *copY* mutation in *S. pneumoniae*, suggesting that CopY may not be the only copper-responsive regulator in *S. pneumoniae* or that these genes may be stimulated indirectly by the presence of copper. Therefore, it remains to be investigated how most of the copper-responsive genes identified by the microarray are regulated.

qRT-PCR was used to confirm data from the global transcript analysis. Figure 5A confirms that expression of the *copY* and *copA* genes is induced in pneumococci grown in the presence of 0.05 mM Cu^{2+} and also demonstrates that, in agreement with the transcriptional profiling, the putative *ctpC, ctpE* and *cutC* genes do not show copper-dependent expression *in vitro*. Thus, it appears that CopA is the main *S. pneumoniae* copper transporter induced under these growth conditions.

The cop *operon is induced in pneumococci isolated from the lungs and nasopharynx but not the blood*

We also investigated the expression of *copY*, *copA*, *ctpC, ctpE* and *cutC* genes in pneumococci recovered from infected mouse tissues. None of the five genes tested showed any difference in expression level in pneumococci isolated from the blood compared with pneumococci grown *in vitro* in Sicard's medium (Fig. 5B). However, all five genes demonstrated induced expression in the lungs (Fig. 5B), with *copA* and *cutC* showing higher levels of expression than the other genes. In addition, both *copA* and *cutC* expression showed an increase in pneumococci isolated from the nasopharynx compared with *in vitro*. These results also show that *copA* expression is significantly higher than *copY in vivo* even though the two genes are in a putative operon, suggesting that the regulation of these genes may be complex. Consequently, these results suggest that CopA and CutC may be important for pneumococcal growth in the nasopharynx, which has not been reported previously.

S. pneumoniae *CopA is required for copper resistance* in vitro *and for growth in the nasopharynx and lungs in a mouse model of pneumonia*

To determine the role of CopA in copper tolerance *in vivo*, a *S. pneumoniae* D39 *copA*$^-$ insertion mutant was constructed and characterized. *In vitro* the *copA*$^-$ mutant showed a growth defect in excess copper concentrations similar to the D39 *ΔcopA* and *ΔcupA copA* strains shown in Fig. 4 (data not shown), therefore the two different *copA* mutant strains have the same *in vitro* phenotype. In a mouse model of pneumonia following intranasal infection it was found that the *copA*$^-$ mutant was less virulent than the D39 parental strain. The median survival time of the *copA*$^-$ mutant infected group (58 ± 7 h) was significantly longer than the wild-type infected group (47 ± 4 h) ($P < 0.01$) (Fig. 6A). While the wild-type could be detected in blood at 8 h after infection, the mutant was only detected 8–12 h post infection (Fig. 6B). However, once in blood, the growth patterns of the strains were similar. The numbers of pneumococci also were monitored in the nasopharynx and lungs at different time points (Fig. 6C and D). In the nasopharynx, the *copA*$^-$ mutant colony counts were less than D39 at 12, 24 and 48 h post infection ($P < 0.01$ for 12 h and $P < 0.001$ for 24 and 48 h). In the lungs, a difference in the numbers of the *copA*$^-$ mutant and the wild-type was only detected at 24 h post infection when the *copA*$^-$ mutant numbers (2.8 ± 0.2) were significantly lower than D39 (4.0 ± 0.1) ($P < 0.05$). These results are consistent with the expression data, which showed that *copA* expression was higher in the

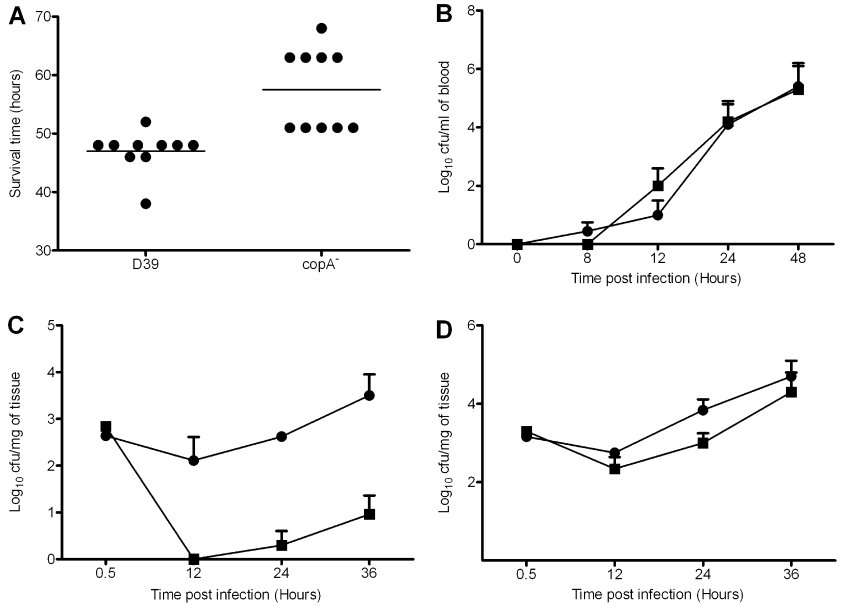

Fig. 6. (A) Survival of mice infected intranasally with D39 or *cop mutant*. Symbols show the time that individual mice became severely lethargic. The horizontal bars mark the median time to the severely lethargic state. Growth of D39 (●) and *copA*⁻ (■) in (B) blood, (C) nasopharynx and (D) lungs. Survival time after intranasal infection with D39 (●) and *copA*⁻ (■). Each datum point derived from 10 mice. Vertical bars indicate SEM.

lungs and nasopharynx. Copper growth assays of wild-type D39 and the *copA*⁻ mutant strains isolated from the mice showed that reversion of the *copA*⁻ mutation and accumulation of secondary mutations had not occurred during passage of the pneumococci *in vivo*, as the passaged bacteria had the same copper resistance phenotype as the bacteria prior to inoculation (data not shown). Therefore, together, these data demonstrate that copper homeostasis is important for pneumococcal survival in the lungs and nasopharynx.

Discussion

In this study, we showed that copper-responsive gene regulation and resistance mechanisms are important for pneumococcal physiology, growth in the nasopharynx and virulence during pneumonia.

Our data showed that the pneumococcal response to copper differs to most other bacteria. Bacteria usually initiate a global adaptive genetic response to copper, which involves induction of other stress regulons (Kershaw *et al*., 2005; Teitzel *et al*., 2006; Ward *et al*., 2008; Baker *et al*., 2010). The pneumococcal copper regulon is surprisingly smaller than many other bacteria as only 39 *S. pneumoniae* genes are differentially expressed in the presence of copper compared with approximately 300 genes in other pathogens such as *Enterococcus faecalis* (Reyes-Jara *et al*., 2010) and *Pseudomonas aeruginosa* (Kershaw *et al*., 2005; Teitzel *et al*., 2006). In addition, unlike other Gram-positive bacteria, such as *Mycobacterium tuberculosis* (Ward *et al*., 2008) and *Staphylococcus aureus* (Baker *et al*., 2010), there is no induction of any genes known to be involved in oxidative stress resistance or the misfolded protein response, showing that *S. pneumoniae* responds differently to copper than other bacteria. This may be typical for lactic acid bacteria because in *Lactococcus lactis* only 14 genes were shown to be copper-regulated (Magnani *et al*., 2008). Therefore, these differences in copper regulons may reflect the physiological requirements and environmental niches of the different bacteria.

Our data show that the *cop* operon is repressed by CopY in low copper conditions. This demonstrates that the pneumococcal CopY protein responds to copper even though it only has the first CxC part of the $CxCx_{4-6}CxC$ copper-binding domain found in other CopY homologues (Portmann *et al*., 2006). The mechanism of CopY copper-responsive repression in *Enterococcus* has been well studied (Portmann *et al*., 2006), and has been shown to be a global regulator (Reyes-Jara *et al*., 2010) as has the *Lactococcus lactis* CopY homologue CopR (Magnani *et al*., 2008). Therefore, it was surprising that in the *S. pneumoniae copY-stop* mutant only the *cop* operon expression was significantly increased compared with the wild-type, suggesting that CopY is not a global regulator in *S. pneumoniae*. The other copper-responsive genes identified by our transcriptional profiling either may be directly regulated by copper via an unknown regulator or are part of other regulons that are stimulated indirectly by the presence of copper.

Bioinformatic analysis of the D39 wild-type genome with the *S. pneumoniae* P*copY cop* box sequence (GACAAATGTA, Fig. 1A) identified only two perfect matches in intergenic regions; the two cop boxes located in P*copY*, which agrees with the microarray data, showing that CopY only regulates the *cop* operon.

Searching the *S. pneumoniae* D39 genome sequence with the *cop* box sequence (GACAAATGTA) allowing 1 mis-match yielded 130 genes. However, none of these genes is regulated by CopY in the microarray analysis. Previous publications have shown that many of the genes identified by motif searches are not CopY-regulated when investigated experimentally (Magnani *et al*. 2008). It may be that the consensus sequence used for the bioinformatic analysis is not the minimum sequence necessary for CopY regulation in *S. pneumoniae* and that more bases may be essential or these genes are not regulated by CopY solely and so are still repressed by other factors under the growth conditions used for the microarray.

Copper is thought to become toxic to bacterial and eukaryotic cells through two major mechanisms: unliganded Cu^{2+} conversion to Cu^{1+} which reacts with H_2O_2 catalysing the generation of highly toxic hydroxyl radicals ($OH^{\bullet}$) and direct interaction of copper with cellular molecules (Arredondo and Nunez, 2005). An important copper toxicity mechanism in *Escherichia coli* is the inactivation of the iron–sulphur clusters of the dehydratase enzymes which leads to defective branched chain amino acid biosynthesis (Macomber and Imlay, 2009). Therefore, most organisms have evolved a number of mechanisms to counteract and prevent further $OH^{\bullet}$ generation as well as limit free copper in the cell.

Copper toxicity is counteracted in *S. pneumoniae* through the action of the *cop* operon. Like other bacteria, our data show that *S. pneumoniae* induces a response to protect the cell from free copper, which involves the CopA ATPase protein. The unique pneumococcal transcriptional response to copper suggests that copper toxicity in *S. pneumoniae* may also involve previously undefined mechanisms. *S. pneumoniae* is already well adapted to oxidative stress as it produces mM levels of H_2O_2 that are toxic to other bacteria (Pericone *et al*., 2003), which may explain the lack of an induced oxidative stress response in the microarray upon exposure to copper. In addition, pneumococci are auxotrophic for several branched chain amino acids due to incomplete biosynthetic pathways (Kazmierczak *et al*., 2009) and have to rely on environmental sources of branched amino acids. This means that pneumococci may be more resistant to copper toxicity mediated through the inactivation of the iron–sulphur clusters of the dehydratase enzymes (Macomber and Imlay, 2009). Interestingly, a number of pneumococcal amino acid transporters are upregulated on exposure to copper, suggesting that there is a requirement for these amino acids in response to the presence of copper possibly due to the toxicity of copper for the biosynthetic pathways of these amino acids.

It is now becoming apparent from our data that copper homeostasis is important for the survival of *S. pneumoniae* in specific host sites. There is increasing evidence that copper is an important metal in the lungs. Copper concentrations are higher in the lungs compared with blood (Catalani *et al*., 2008). Concurrent with this, our data show increased expression of *copY, copA, cutC, ctpE* and *ctpC* and the attenuation of virulence of a *copA*$^-$ mutant in the lungs but not in blood. In addition, signature tagged mutagenesis identified CopA as well as CtpE and CutC as being important for pneumococcal infection of the lung (Hava and Camilli, 2002). Copper also appears to be important for the behaviour of pneumococcus in the nasopharynx as there is increased expression of *copA* and *cutC*, and decreased survival of the *copA*$^-$ mutant in the nasopharynx in this mouse model of acute invasive disease. CutC is conserved in other *Lactobacillale* species, suggesting that it may have an important role in copper homeostasis (Reyes *et al*., 2006). The decrease in the *copA*$^-$ mutant bacterial count in the nasopharynx could be due to the bacteria being unable to adhere to the cells or mucus of the nasopharynx, or the *copA*$^-$ mutant bacteria may be unable to exploit the environment and grow in the nasopharynx. The importance of copper resistance for virulence has been shown for other respiratory pathogens, including *Mycobacterium tuberculosis* (Wolschendorf *et al*., 2011) and *Pseudomonas aeurginosa* (Schwan *et al*., 2005). However, the importance of bacterial copper homeostasis in the nasopharynx has not been described previously and requires further investigation.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains used for this study are listed in Table S1. Pneumococci were grown as static cultures in M17 (Terzaghi and Sandine, 1975) broth containing 0.5% (w/v) glucose (GM17) or CDM containing 0.5% (w/v) glucose at 37°C in air or in brain heart infusion (BHI) broth or on blood agar plates supplemented with 1% (v/v) defibrinated sheep blood or 5% (v/v) defibrinated horse blood, in microaerophilic conditions at 37°C. CDM was prepared as described before (Kloosterman *et al*., 2006a), with the exception that $ZnSO_4$ and $CuSO_4$ were omitted from the metal mixture and added separately as specified in *Results*. Metal ions were added as the salts $ZnSO_4$, $MnSO_4$, $MgCl_2$, $CaCl_2$, $CoCl_2$, $NiSO_4$, $CuSO_4$ and $FeCl_2$. For growth experiments, β-galactosidase assays and transcriptome analysis, *S. pneumoniae* cells frozen at an optical density at 595 nm of 0.3 in GM17/CDM were washed once with the appropriate medium and diluted 1:40 for inoculation. To determine the copper susceptibility of the *copA*$^-$ mutant strain, Sicard's defined medium (Sicard, 1964) was supplemented with 0–2.5 mM Cu^{2+} and the increase in optical density at 500 nm was determined every hour over 16 h. *Escherichia coli* strain EC1000 was cultured at 37°C on TY agar or TY broth. Where necessary for selection, media were supplemented with the following concentrations of

antibiotics: erythromycin: 0.25 μg ml^{-1} for *S. pneumoniae* and 120 μg ml^{-1} for *E. coli*; spectinomycin: 100 μg ml^{-1} and tetracycline: 2.5 μg ml^{-1} for *S. pneumoniae*; and ampicillin: 100 μg ml^{-1} for *E. coli*. When appropriate, 0.006% (w/v) X-Gal was used in plates. Long-term storage of bacteria was done at –80°C in 10% (v/v) glycerol.

DNA isolation and manipulation

Primers used in this study are listed in Table S2. Chromosomal DNA of *S. pneumoniae* D39 wild-type strain was used as a template for PCR amplification (Avery *et al*., 1944; Lanie *et al*., 2007). All DNA manipulations were done as described before (Kloosterman *et al*., 2006a).

Construction of mutants

The *copY-stop* and *cupA-stop* mutants were constructed using plasmid pORI280 as described before (Kloosterman *et al*., 2006b). Briefly, primers SPD0633-2 NcoI/SPD0633-3 NcoI and SPD0634-2 NcoI/SPD0634-3 NcoI, which introduce four premature stop codons and a NcoI site in frame after the first 6 bases in the *copY* (SPD0663) and 21 bases in the *cupA* (SPD0634) open reading frame, were used in combination with primers SPD0633-1 XbaI and SPD0633-4 EcoRI and SPD0633-1 BglII and SPD0633-4 XbaI respectively, to amplify fragments comprising the upstream and downstream sequence of *copY* (SPD0633) and *cupA* (SPD0634). These PCR products were ligated using the NcoI site. The resulting products were cloned respectively as an XbaI, EcoRI, or BglII, XbaI fragment in pORI280, giving plasmids pSS1 and pSS2. The mutations led to the appearance of an NcoI site, on the basis of which the desired mutant could be identified. The mutations were further verified by DNA sequencing. pSS1 and pSS2 were used to introduce the mutations into the chromosome of *S. pneumoniae* D39 as described before (Kloosterman *et al*., 2006a), giving the *copY-stop* and *cupA-stop* mutant strains.

Deletion strains of *copA* and *cupA-copA* were made with allelic replacement with a spectinomycin marker. Briefly, primers SPD0634-1/SPD0634-2, SPD0635-1/SPD0635-2 and SPD0635-3/SPD0635-4 were used to generate the PCR fragments of the left and right flanking regions of *copA* and *cupA-copA* respectively. Then these PCR products were ligated using AscI/NotI site with the spectinomycin PCR product, which was generated with primers Spec-F/Spec-R. The ligated product was transferred to *S. pneumoniae* D39. Spectinomycin resistance clones were examined for the presence of the *copA* or *cupA-copA* deletion by PCR.

To construct the *copA*$^-$ insertion mutant, the chromosomal region encompassing *copA* (SPD0635) was amplified with copAF and copAR primers (Table S2). The amplicons were incubated with *Himar1* transposase (Lampe *et al*., 1996) and plasmid pR412, which contains the *mariner* mini-transposon conferring spectinomycin resistance (Martin *et al*., 2000). Then the *in vitro* mutagenized DNA was transformed into the pneumococcus (Alloing *et al*., 1996). Pneumococcal transformants were selected for spectinomycin resistance, and insertion of the resistance cassette into the chromosome was confirmed by PCR by using transposon-specific primers, MP127 or MP128, with appropriate chromosomal primers, and sequenced as described previously (Yesilkaya *et al*., 2009). A representative strain, designated as *copA*$^-$, was selected for further study.

Construction of lacZ fusions

Chromosomal transcriptional *lacZ* fusions to the P*copY* (whole *copY* promoter region), P*copY-F* (cop box 1 only) and P*copY-C* (cop box 1 and 2) were constructed in the integration plasmid pPP2 (Halfmann *et al*., 2007) via double crossover in the *bgaA* gene with primer pairs FcopY/RcopY, FcopY/PcopY-F and FcopY/PcopY-C leading to plasmids pSS3-5 respectively. The *lacZ* fusion constructs were introduced into wild-type D39, as well as the D39 *copY*-stop mutant as described above. All plasmid constructs were checked by sequencing, and new loci created with these plasmids were verified by PCR.

Complementation of copY

To complement the *copY* gene in the D39 *copY*-stop mutant, we amplified the native *copY* promoter and gene with primer pair FcopY and PcopY-com. This PCR product was further cloned into pPP2 plasmid (Halfmann *et al*., 2007) resulting in a plasmid pSS4. pSS4 was transferred to D39 *copY*-stop mutant strain to complement the *copY* gene.

β-Galactosidase assays

β-Galactosidase activity was measured as described before (Hava and Camilli, 2002) using cells grown in CDM at 37°C supplemented with different concentrations of metal ions as mentioned in *Results* and harvested in the mid-exponential phase of growth.

Transcriptome analysis using S. pneumoniae *DNA microarrays*

DNA microarrays were used to determine Cu^{2+}-dependent pneumococcal gene expression (Kloosterman *et al*., 2006b). The expression in D39 strain grown in CDM supplemented with 0.05 mM $CuSO_4$ was compared with the transcriptome of the same strain in the absence of Cu^{2+}. The experiments were repeated with four biological replicates essentially as described previously (Kloosterman *et al*., 2006b; van Hijum *et al*., 2005). In short, cultures were harvested at an optical density (OD) at 595 nm of approximately 0.3 by centrifugation for 1 min at 10000 r.p.m. at room temperature. Cell pellets from 50 ml culture for each replication were immediately frozen in liquid nitrogen and store at –80°C. RNA was isolated with the Roche RNA isolation kit. Synthesis of cDNA and Cy3/Cy5 labelling of 15–20 μg total RNA was performed with the CyScribe Post Labelling Kit (Amersham Bioscience). Hybridization was performed with labelled cDNA for 16 h at 45°C in Ambion Slidehyb #1 hybridization buffer on superamine glass slides (Array-It, SMMBC). Slides were scanned with a Genepix 4200 laser scanner at 10 μm resolution. Array

Pro 4.5 (Media Cybernetics, Silver Spring, MD, USA) was used to analyse the slides. The *MicroPrep* software package was used to obtain the microarray data from the slides. The expression ratio of D39 strain + 0.05 mM Cu^{2+} over the D39 strain + 0 mM Cu^{2+} was calculated from the measurements of at least 7 spots by Cyber-T.

For transcriptome analysis of D39 wild-type strain and its isogenic *copY* mutant, cells were grown in CDM without Cu^{2+} and harvested at an optical density at 595 nm of approximately 0.3. The experiments were repeated with four biological replicates. All other procedures regarding microarray were done as described above. Microarray data have been deposited to the Gen Expression Omnibus (GEO), and can be accessed via GSE30415 (www.ncbi.nlm.nih.gov/geo/).

Inductively coupled plasma- mass spectrometry (ICP-MS) analysis

For ICP-MS analysis, samples were prepared as describe before (Shafeeq *et al.*, 2011). In short, cultures of D39 wild-type and *copA* mutant were grown in 100 ml of CDM with and without 0.05 mM Cu^{2+}. Cultures were centrifuged and washed (at 4°C) once with the CDM medium and twice with phosphate-buffered saline (PBS) that had been treated with chelex (Sigma) overnight. The cell pellets were dried overnight in a Speedvac. The dried cells were subsequently used for analysis by means of ICP-MS, as described before (Jacobsen *et al.*, 2011). Results were expressed as μg of Cu^{2+} per g dry weight of cells.

RNA extraction, RT-PCR and purification for quantitative RT-PCR

Total RNA was extracted from *S. pneumoniae* strain D39 grown to mid-log phase in Sicard's defined medium (Sicard, 1964) in the presence or absence of 0.05 mM Cu^{2+} in microaerophilic conditions. Bacteria were harvested by centrifugation at 3000 *g* for 10 min. RNA was extracted by TRIZOL method as described by the manufacturer (Invitrogen, Paisley, UK), and purified by RNeasy purification kit (Qiagen, Crawley, UK). RNA was quantified and its integrity was checked by ethidium bromide staining after electrophoresis through a 1% (w/v) agarose gel. Any contaminating DNA was removed by treatment with 2 U RNase-free DNase I (Invitrogen, Paisley, United Kingdom) for 15 min at room temperature, followed by heat inactivation for 10 min at 65°C in the presence of 2.5 mM EDTA.

First strand cDNA synthesis was performed on approximately 1 μg DNase-treated total RNA, immediately after isolation, using 200 U of SuperScript II reverse transcriptase (Invitrogen) and random hexamers at 42°C for 55 min (Yesilkaya *et al.*, 2008). cDNA (2 μl) was amplified in a 20 μl reaction volume that contained 1 × SYBR Green PCR master mix (Applied Biosystems, Foster City, USA) and 3 pmol of each primer (Table S2). The transcription level of specific genes was normalized to *gyrB* transcription, amplified in parallel with SP0806F and SP0806R primers. The reactions were performed in triplicate using the following cycling parameters: 1 cycle of 10 min 95°C followed by 40 cycles of 30 s 95°C, 1 min 55°C, and 30 s 72°C. The results were interpreted using the comparative C_T method (Schmittgen and Livak, 2008). Differences in expression of twofold or greater relative to control were considered as significant. To confirm the polycistronic nature of the *cop* operon total RNA was isolated from *S. pneumoniae* D39 wild-type grown in CDM + 0.05 mM Cu^{2+}. Primers copY-F and cupA-R were used to amplify the IR-I intergenic region between copY and *cupA*, whereas the IR-II intergenic region between *cupA* and *copA* was amplified using primers cupA-F and copA-R. Primers are listed in Table S2. PCRs were performed with 1/100 part of the RT reactions, and 200 ng of RNA and 45 ng DNA.

Extraction of pneumococcal RNA from infected tissues

Outbred 8- to 9-week-old female MF1 mice (Harlan Olac, Bicester, UK) were intranasally infected with 50 μl PBS containing 1×10^6 passaged type 2 pneumococcal, as before (Yesilkaya *et al.*, 2000). When the mice became severely lethargic they were anaesthetized and blood (0.5–1 ml) was collected by cardiac puncture. After killing by cervical dislocation, the lungs were removed and homogenized on ice in 10 ml sterile PBS using a tissue homogenizer. The pneumococcal mRNA was extracted from approximately 250–300 mg infected mouse lung tissue samples. The nasopharynx (40–60 mg per mouse) was dissected by removing the entire palate. Then nasopharyngeal tissues were transferred into sterile PBS and homogenized. To separate pneumococci from host cells, lung homogenates and blood samples were centrifuged at 900 *g* for 6 min at 4°C. Supernatants were subsequently centrifuged at 15 500 *g* for 2 min at 4°C, and the bacterial pellet was stored at –80°C until further processing. Prior to pelleting, 20 μl homogenate was removed, serially diluted in PBS and plated onto blood agar in order to enumerate pneumococci and to exclude the presence of contaminating microorganisms. RNA extraction and purification were done as described in the previous section, and routinely 0.8–3 μg total RNA per sample could be obtained.

In vivo *virulence studies*

Ten-week-old female MFI outbred mice (Harlan Olac) were used for virulence testing. A standardized inoculum was prepared as described previously (Yesilkaya *et al.*, 2000; 2006). To determine the virulence of pneumococcal strains, mice ($n = 10$ for each group) were infected intranasally with approximately 1×10^6 *S. pneumoniae* cfu as described before (Yesilkaya *et al.*, 2000; 2006). The inoculum dose was confirmed by viable counting on blood agar plates. Mice were monitored for disease signs (progressively starry coat, hunched and lethargic) for 7 days, and those that reached the severely lethargic stage were considered to have reached the end-point of the assay and were killed humanely. The time to this point was defined as 'survival time'. Mice that were alive 7 days after infection were deemed to have survived the infection. To determine the development of bacteraemia in each mouse, approximately 20 μl venous blood was obtained from intranasally infected mice at predetermined time points after infection. Viable counts in blood were determined by

serial dilution in sterile PBS and plating onto blood agar plates (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood with appropriate antibiotic. Survival times were analysed by the Mann–Whitney *U*-test. Growth of pneumococci in the nasopharynx and lungs was also determined at 0.5, 12, 24 and 36 h post infection. For this, at predetermined time intervals following intranasal infection, set groups of mice ($n = 5$ for each time point) were deeply anaesthetized as before and subsequently the mice were killed by cervical dislocation. The lungs and nasopharynx were transferred separately into 10 ml of sterile PBS, weighed and homogenized (Yesilkaya *et al*., 2000). Viable counts in homogenates were determined as above. Data were analysed by analysis of variance followed by the Beferroni post-test.

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